1981

Characterization of insulin and growth hormone receptors in bovine tissues

Steven Lynn Nissen
Iowa State University

NISSEN, STEVEN LYNN

CHARACTERIZATION OF INSULIN AND GROWTH HORMONE RECEPTORS IN BOVINE TISSUES

Iowa State University PH.D. 1981

PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark __√__.

1. Glossy photographs or pages _____
2. Colored illustrations, paper or print _____
3. Photographs with dark background _____
4. Illustrations are poor copy _____
5. Pages with black marks, not original copy _____
6. Print shows through as there is text on both sides of page _____
7. Indistinct, broken or small print on several pages _____
8. Print exceeds margin requirements _____
9. Tightly bound copy with print lost in spine _____
10. Computer printout pages with indistinct print __✓__
11. Page(s) _____ lacking when material received, and not available from school or author.
12. Page(s) _____ seem to be missing in numbering only as text follows.
13. Two pages numbered _____. Text follows.
14. Curling and wrinkled pages _____
15. Other _____

University
Microfilms
International

Characterization of insulin and growth hormone receptors in bovine tissues

by

Steven Lynn Nissen

A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department: Animal Science
Major: Animal Nutrition

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa
1981

TABLE OF CONTENTS

GENERAL INTRODUCTION

The importance of hormones in regulating animal metabolism and growth is usually judged by observing changes in plasma hormone concentration coincidental with changes in metabolism or growth pattern. This approach has been useful in describing the endocrinology of reproduction largely because of the dramatic changes in hormone concentration associated with reproductive physiology. The endocrine changes associated with changes in animal metabolism are small, however, necessitating an exact description of the animal's hormone status if the relationship between hormones and growth are to be understood.

Conceptually the "hormonal status" of an animal is thought to depend on both the concentration of the hormone at the tissue level and the responsiveness or sensitivity of the tissue to the given hormone concentration. Presently the techniques of radioimmunoassay adequately measure circulating hormone concentrations, but tissue hormone sensitivity is less directly measurable.

Tissue hormone sensitivity is determined by at least two separate events; the actual binding of hormone to a specific cell surface receptor and subsequent initiation of cellular events responsible for the ultimate hormone action. Of the two, hormone binding is technically easiest to measure <u>in vitro</u>. Thus, receptor binding has gained popularity as a measure of hormone sensitivity, realizing postreceptor events may also greatly affect hormone sensitivity.

Hormone binding is composed of two components: hormone affinity and receptor number. The product of these is proportional to the amount of hormone that will bind at a given hormone concentration. Thus, by knowing the binding characteristics of a tissue and the hormone concentration, an estimation of the amount of hormone bound can be made. This should relate to biological actions of the hormone, keeping in mind possible postreceptor modulation of sensitivity.

Cattle growth and metabolism is thought to be influenced by hormones, particularly insulin and growth hormone. To date, only plasma hormone concentrations have been related to changes in growth. It appears that lean and rapid growth is associated with low insulin concentrations and high growth hormone concentrations. It is, however, unknown if these hormone changes result in changes in hormone action. A simultaneous measure of hormone sensitivity must be made to properly evaluate the importance of the hormones in growth and metabolism--in this case, receptor binding.

The studies presented here examine the physical aspects of insulin and growth hormone binding in various cattle tissues, as well as comparing binding in cattle of large and small frame sizes. These data should further the understanding of the actions of insulin and growth hormone on cattle growth.

This dissertation consists of two journal papers as they will be submitted for publication. The general introduction and general discussion serve to deal with concepts not covered in the paper. The Appendix contains detailed descriptions of methods used in hormone binding assays, as well as supplemental data not contained in the main body of the thesis.

SECTION I. INSULIN AND GROWTH HORMONE RECEPTORS IN CATTLE TISSUES: CHARACTERIZATION AND COMPARISON WITH RAT TISSUES

Steven Nissen and Allen Trenkle

Department of Animal Science,

Iowa State University,

Ames, Iowa 50011

ABSTRACT

Binding of [^{125}I] bovine insulin and [^{125}I] bovine growth hormone (bGH) to mononuclear cells isolated from blood and plasma membranes isolated from liver, kidney and fat was characterized in cattle and rats. Insulin and bGH receptors from rats and cattle exhibited specificity and high affinity (Ka_1 5 to 10 x 10^8 M^{-1} and Ka_2 2 to 20 x 10^7 M^{-1}). Bovine mononuclear cells had approximately 3,700 high affinity and 15,000 low affinity insulin receptors per cell. All bovine tissue membranes examined exhibited similar insulin binding characteristics, but maximum binding percentage was only 1/3 that of rat tissues. Rat liver and adipose tissue membranes bound similar amounts of insulin, while kidney bound only 1/10 of that by liver and adipose tissue. bGH did not bind specifically to bovine mononuclear cells. bGH binding to liver membranes of rats and cattle was similar. Rat kidney membranes specifically bound bGH but specific binding could not be demonstrated in bovine kidney. This study indicates insulin and bGH receptors in rats and cattle are physically similar but some major organ differences exist between the two species, such as those observed in kidney.

INTRODUCTION

Evidence relating the effect of peptide hormones to the amount of hormone specifically bound to cell surface receptors (Anderson et al., 1977; Kono and Barham, 1971; Olefsky, 1976) suggests that a responsiveness of a tissue to a given hormone concentration could be predicted if the binding characteristics of that tissue is measured. Thus, physical characterization of hormone receptors coupled with the plasma hormone concentrations should give an estimate of the hormone specifically bound to a tissue. Although it is thought that insulin receptors are very similar between species (Kemmler et al., 1978; Muggeo et al., 1979), insulin and growth hormone binding have not been studied in bovine tissues. Additionally, the techniques for isolation of bovine mononuclear cells and tissue plasma membranes have not been described.

The objective of this work was to characterize and compare insulin and growth hormone receptors in bovine mononuclear cells and isolated plasma membranes from liver, kidney, and adipose tissue. As a comparative control, binding to rat tissues was also measured.

MATERIALS AND METHODS

Hormone and tissue preparation

Bovine insulin (Calbiochem), chicken insulin (Lilly), bovine proinsulin (Lilly), bovine insulin A chain (Sigma) and bovine insulin B chain (Sigma) were prepared from powder just prior to use and dissolved in .01N HCl. Bovine growth hormone (gGH, Li 1954), porcine growth hormone (pGH, Papkoff et al., 1962), ovine prolactin (oPRL, Cole and Li, 1955), and bovine prolactin (bPRL, Cole and Li, 1955) were kindly provided by Dr. C. H. Li. Ovine growth hormone (oGH) was prepared by the method of Wallace and Ferguson (1963) and further purified by chromatography on Sephadex G-150 in pH 9.1, in .01 M sodium bicarbonate buffer. Rat growth hormone (rGH) was obtained from the Pituitary Distribution Program of NIAMDD. Glucagon (Lilly) and growth hormones were dissolved in normal saline. Diethylstilbesterol (Merck) and triiodothironine (Sigma) were dissolved in .01N NaOH while estradiol (Calbiochem) was taken up in 80% ethanol. Hormones were further diluted in 25 mM assay buffer before use in binding studies (see below).

[^{125}I] bovine insulin and [^{125}I] bovine growth hormone were prepared by the stoichiometric iodination procedure outlined by De Meyts (1976a). The only modification was that the level of [^{125}I] incorporation into bGH was decreased from 40 to 25% of the total. This lower specific activity of bGH was necessary to retain binding activity and presumably biological activity. Iodinated insulin was isolated by adsorption chromatography on a cellulose column while iodinated bGH was separated from unincorporated iodine by chromatography on a Sephadex G-100 column in 25 mM assay

buffer (AB: MOPS 25 mM, NaCL 105 mM, KCl 5 mM, $MgSO_4$ 1.2 mM, NaAcetate 15 mM, glucose 6 mM, EDTA 1 mM, and bovine serum albumin 1 mg/ml, pH 7.4). Aliquots (100 µl) of the iodinated hormones were stored at -20 C until needed. In all cases, the labeled hormones were used within 30 days of iodination. Just prior to use, the labeled insulin was repurified on Sephadex G-50 and labeled bGH was repurified on Sephadex G-150. Again 25 mM AB was used as solvent. Both hormones were diluted to approximately 10^4 CPM/50 µl. Fifty µl of the labeled hormone were used in the binding assays. The final [^{125}I] insulin concentration in the medium was calculated to be approximately 0.3 ng/ml, while the final [^{125}I] bGH concentration was approximately 0.6 ng/ml. Precipitability of both preparations was greater than 95% in 10% trichloroacetic acid.

Blood was obtained from cattle at the time of slaughter to isolate mononuclear cells. Three hundred mls of blood were collected directly into a flask containing 20 ml of isotonic EDTA (4.6 g/100 ml) and 20 ml of isotonic morpholinopropanesulfonic acid (MOPS; Sigma; 3.68 g/100 ml, adjusted to pH 7.4 with NaOH). This mixture provided sufficient anticoagulant to prevent platelet aggregation within the buffy coat after centrifugation. The blood-buffer-EDTA mixture was cooled to 10-15 C for transportation to the laboratory. Two hundred fifty mls of the blood mixture were centrifuged at 1500G for 15 minutes in a swinging bucket rotor. The buffy coat was removed and diluted in 25 ml of 25 mM AB. The measured osmolarity of this buffer was 280 mOsm/l. The mononuclear cells were then isolated by a modification (DeMeyts, 1976a) of the density gradient method

of Boyum (1968). Twenty to 25 ml of the buffy coat suspension were carefully layered over 10 ml of a 1.075 g/ml Ficoll-Hypaque gradient. The gradient composition was as follows: 56 ml of 17.4% Ficoll (w/v; Pharmacia), 12 ml of Hypaque-50% (Winthrop Laboratories), 24 mls of 25 mM AB and 6 ml of distilled water. This mixture was isotonic to bovine plasma (280 mOsm/l) and gave superior separation of mononuclear cells than the 1.079 g/ml gradient used by Boyum with human blood. The gradient was centrifuged for 40 minutes at 400 G at 18 C. The white mononuclear cell layer (just above red cells) was then removed and diluted to 30 mls with AB. This suspension was then centrifuged 16 minutes at 250 G at 5 C. The cells were suspended in 25 mM AB and centrifuged at 250 G at 5 C for eight minutes two additional times to remove platelets.

The final washed cell pellet was suspended in 5 to 15 ml of 100 mM AB (MOPS concentration increased to 100 mM and NaCl decreased to 40 mM to maintain isotonicity) depending on the pellet size. An approximation of cell numbers was obtained by counting in a hemocytometer. Cells were diluted to approximately 2.5×10^7 cells/ml with 100 mM AB. This cell suspension was then used for insulin binding assays.

Final cell concentration and viability (trypan blue exclusion) were measured in a hemocytometer. Cells were also counted on a Coulter Counter (Coulter Electronics) equipped with a cell size channelizer. From the size distribution plots, the percent of large and small cells in each preparation was estimated. The bimodal distribution of cell sizes was confirmed by microscopic examination. Other attempts to quantitate cell

types in the bovine mononuclear cell preparations proved unsatisfactory due to cell breakage (esterase stain, DeMeyts, 1976a) and cell clumping (phagocytois of latex beads, DeMeyts, 1976a). Thus, the cell size channelizer method of estimating large "type" cells was used as an indication of the monocyte population, as monocytes are the most numerous large cells present.

Illustrated in Figure 1 is a typical size profile of bovine mononuclear cells prepared from Ficoll-Hypaque gradients. The major peak (channel 35-50), contained primarily small lymphocytes, while the larger cells were included in the second minor peak (channel 50-65). Most preparations contained 20-35% large type cells. Red blood cells were in channel 20-30 while platelets and broken cells were in channel 0-20. Changing the dilution of cells counted did not increase the percentage of large cells as would be expected if coincidental counting of two cells were responsible for the large cell peak. Since specific data describing differential insulin binding to bovine lymphocytes and monocytes are not available, the binding data were expressed on the basis of total mononuclear cells.

Bovine liver, kidney and mesenteric adipose samples were removed within an hour after slaughter, frozen and stored at -20 C until plasma membranes could be isolated. Samples of rat liver, kidney and epididymal fat were collected and treated similarly. The plasma membranes were isolated by a procedure outlined by Hollenberg and Cuatrecasas (1976).

Briefly, 15 g of tissue were homogenized in 150 ml of .25 M sucrose with a Polytron homogenizer (Brinkman Instrument; power setting of 3.2) for 90 seconds. After centrifuging at 600 G for 10 minutes, the supernatents were transferred to 50 ml tubes and centrifuged at 10,000 G in a fixed angle centrifuge for 30 minutes. Floating fat material was removed by aspiration and supernatants were transferred to clean 50 ml tubes and the volume adjusted to 40 ml with .25 M sucrose. One ml of 23.4 g/100 ml NaCl (.1 M final) and .5 ml of .193 g/100 ml $MgSO_4$ (.2 mM final) were added and centrifuged for 45 minutes at 37,000 G in a fixed angle centrifuge. Supernatants were discarded and the membrane pellet was washed and centrifuged three times with 100 mM $NaHCO_3$. The final pellet was suspended in 5 mM $NaHCO_3$, analyzed for protein (Lowry et al., 1951) and stored at -20 C for use in the binding studies. Approximately 200 mg of membrane protein were isolated from the original 15 g of liver and kidney. Membrane recovery from fat tissues was much lower.

Animals

Fourteen steers, primarily of Angus and Simmental breeds were fed a high energy diet (85% corn grain) adequate in minerals, vitamins and protein. At the time of slaughter, the cattle were 11-13 months of age and weighed approximately 400 kg. Twenty male rats of Sprague-Dawley descent were maintained on a commercial diet and weighed approximately 150 g. Both cattle and rats were fed ad libitum and tissues were collected between 0800 and 1000 hours.

Binding assays

Mononuclear cells were suspended in 100mM AB while membranes were suspended in 25mM AB plus 15 mg/ml BSA for binding assays. All binding studies were carried out in polystyrene tubes (10 x 75 mm, Falcon Plastics).

Insulin and GH binding studies were conducted by making the following additions to each tube: 50 µl of labeled hormone preparation, 50 µl of unlabeled hormone (0 to 10,000 ng/ml) and 500 µl of cell or membrane suspension. Insulin and bGH binding at 20 C reached steady state at approximately 6 hours of incubation, at which time duplicate aliquots (200 µl) were layered over 200 µl of ice cold 25mM AB in 500 µl polyethelene microfuge tubes (Sarstedt).

Cell suspensions were spun for one minute (Beckman microfuge) and membranes were spun for 1.5 minutes. Tubes were inverted, and by a quick snap of the wrist, the supernate and pellet were completely separated by an air bubble. While still inverted, the microfuge tips were cut off to obtain bound counts while the free counts were obtained by counting the remaining portion of the tube. Although all suspensions of membranes assayed were diluted to contain 200 µg protein/ml, measurements of the pellet protein were made (Lowry et al, 1951) and the quantity of labeled hormone bound was corrected for the actual amount of protein spun down in the microfuge tube. Hormone degradation was measured by exposing the supernate to an equal volume of 10% TCA and determining the number of counts precipitated by the TCA. In most cases degradation was $< 10\%$ in both mononuclear cell and membrane assays. No attempt was made to correct for hormone degradation.

Calculations

Specific hormone binding was calculated by subtracting the counts bound in the presence of 10,000 ng/ml cold hormone from the total binding. GH binding studies yielded linear Scatchard (1949) plots while Scatchard plots for insulin binding were curved. The curved Scatchard plots were analyzed by two models:

I. Negative cooperative model; after Kahn et al., (1978).

$$B/F = \frac{Ke}{1 + \frac{1-a}{a}} \times (Ro-B)$$

II. Two class model; after Feldman (1972).

$$B/F = .5 \times K_a1 \times (R_o1-B) + K_a2 \times (R_o1-B) + \sqrt{K_a1 \times (R_o1-B) - K_a2 \times (R_o2-B)^2}$$

Where:

B/F = specific counts bound/counts free

B = hormone specifically bound (ng or Moles)

Ke = average affinity of empty site (M^{-1})

a = alpha factor or interaction factor (Ke/Kf)

Ro = total receptor number (ng)

Ka = affinity association constant for one class model (M^{-1})

Ka1 = high affinity association constant (M^{-1})

Ro1 = number of high affinity receptors (ng)

Ka2 = low affinity association constant (M^{-1})

Ro2 = number of low affinity receptors (ng)

Two models were used to analyze curvilinear binding data because it is not certain whether the curvilinear Scatchard plot is due to interaction between binding sites (DeMeyts, 1976b) or due to the presence of more

than one class of independent receptors (Olefsky and Chang, 1978). Both models were analyzed by a least squares iterative procedure (Barr et al., 1979) using equations I and II described above.

RESULTS

The curves depicting time course binding of labeled insulin to bovine liver membranes and mononuclear cells and bGH binding to bovine liver membranes are illustrated in Figure 2. All other rat and bovine tissues also reached maximum within 6 hours. Varying the concentration of membrane (150 to 500 μg/ml) and cell number (5 to 75 x 10^6 cells/ml) resulted in a linear increase in maximum binding percent. Other studies of insulin and bGH binding at 38C indicated faster initial binding but with lower steady state binding than obtained at 20C.

Data showing competition of unlabeled hormones with labeled bovine insulin for binding to bovine liver membranes are presented in Figure 3a. Chicken insulin was equal to bovine insulin in displacing labeled insulin. Bovine proinsulin displaced labeled insulin from its receptors but was much less active than bovine insulin. There was no competition of oGH and glucagon. In other studies with mononuclear cells, kidney and fat membranes, competition curves were qualitatively similar to that shown in Figure 3.

Competition of hormones with labeled bGH for binding to bovine liver membranes is presented in Figure 3b. With bovine liver, oGH and bGH were similar in their ability to displace [^{125}I] bGH from its receptor while porcine and rat GH were somewhat less potent in displacing bGH. Both oPRL and bPRL were approximately 100 times less potent than bGH. Other peptides and steroids did not affect bGH binding.

Scatchard plots of [^{125}I] insulin binding to liver, kidney and fat membranes of rats and cattle are shown in Figure 4. Rat liver bound approximately twice as much insulin as bovine liver. The higher binding by the rat liver membranes was related to an increase in the number of low and high affinity receptors. Negative cooperative analysis resulted in Ro(ng), Ke (x 10^8 M^{-1}) and alpha values of 4.4, 4.2 and .022 for bovine and 14.2, 3.4 and .091 for rat membranes.

The bovine kidney (Figure 4b) bound 3 times more insulin than rat kidney. Analysis on the basis of two independent receptors indicated the increased binding was due to greater number of receptors, whereas the negative cooperative model indicated the differences were due primarily to changes in the Ke and alpha: Ro(ng), Ke(x 10^8 M^{-1}) and alpha values for bovine were 1.13, 9.0, .068 and for rat were 1.81, 2.0 and 0.16.

Insulin binding to adipose membranes is also illustrated in Figure 4c. As with liver, insulin binding to rat adipose membranes was greater than bovine. A greater number of receptors appeared to be responsible for the higher binding. The negative cooperative model indicated that changes in the Ke were responsible for the differences in adipose binding. Binding parameters Ro, Ke and alpha in cattle were 5.0, 1.9, .0038, respectively, while the parameters in rats were 2.3, 15.8 and .311, respectively.

Insulin binding to bovine mononuclear cells is presented in Figure 5. Binding to mononuclear cells contrasted to isolated membranes, in

that a much larger portion of the insulin was bound to low affinity receptors.

Binding of bGH to plasma membranes of rat and bovine liver and kidney is shown in Figure 6. The maximum amounts of bGH bound were approximately equal in both rat and bovine liver membranes, but the Ro and Ka were much different. The rat liver membranes had a high capacity low affinity receptor while the bovine liver had a high affinity low capacity receptor. Specific binding of labeled bGH to bovine kidney membranes could not be demonstrated.

Specific GH binding was also noted in some adipose membrane preparations but insufficient membrane was available to fully characterize the binding. Attempts to measure displacement of [^{125}I] GH from mononuclear cells resulted in an increase in total binding as more cold GH was added. The failure of GH to specifically bind to mononuclear cells has been demonstrated before (Hinterberger et al., 1976).

DISCUSSION

It has been suggested that unlike the primary amino acid sequence of insulin, the insulin receptor is conserved phylogenetically (Kemmler et al., 1978, Muggeo et al., 1979). Competition studies and affinity measurements reported in the present study support the concept that the bovine insulin receptor is very similar to that of rat (Cuatrecasas, 1971), turkey (Ginsberg et al., 1976), and human (Olefsky et al., 1976).

The data obtained in this study of insulin binding to rat liver membranes are comparable with published studies analyzed as a negative cooperative model (Kahn et al., 1978). When the data were analyzed on the basis of two binding sites, the affinities were slightly lower than reported elsewhere for human and rat (Gavin et al., 1973, Kahn and Roth, 1976). Other reports of insulin binding to bovine liver membranes (Rosen et al., 1979) indicated similar affinities and binding capacities to those reported here. Isolated ovine hepatocytes (Gill and Hart, 1980) had slightly higher affinities for insulin than measured here for bovine liver membranes. The least squares analysis employed in our study may account for the somewhat lower affinity, but more likely it is that other reports failed to properly analyze the contribution of each receptor class in the Scatchard analysis (Feldman, 1972; Norby et al., 1980).

Data comparing insulin binding to bovine and rat tissues are limited. Takano et al., (1976) reported similar insulin binding in rat and bovine liver membranes. Rosen et al., (1980) also indirectly compared binding of rat and bovine liver, noting similar binding except that the addition of cold insulin did not enhance the displacement of labeled insulin.

Although liver membranes from rats bound more insulin than those from cattle, attempts were not made to evaluate if this difference could be attributed to differences in membrane purity. If impurity of the membranes caused the low binding of insulin to bovine liver, it would be expected that binding of bGH also would have been low. The bovine liver membranes, however, bound bGH equal to rat membranes, suggesting that gross changes in membrane purity were not responsible for the lower insulin binding to bovine liver membranes.

The bovine mononuclear cells had approximately 3,700 high affinity and 15,000 low affinity insulin receptors per cell, which is somewhat higher than reported for human cells (Olefsky et al., 1976), while the affinity of the bovine mononuclear cells for insulin is somewhat lower than reported for the human (Gavin et al., 1972; Gavin et al., 1973). Again, the lower affinity reported here may be due to differences in the mathematical analysis employed.

The studies reported here give little insight into whether the curved Scatchard plot is due to negative cooperativity or the presence of two classes of insulin receptors. Indeed, there is evidence to suggest that there may be functionally two classes of receptors of which one or more interact, causing the negative cooperative effects noted (Olefsky and Chang, 1978). Whatever model is eventually accepted, it makes little difference when affinities and receptor numbers are used for comparative purposes and not for physiochemical inferences into receptor-hormone interaction.

Initial attempts at demonstrating bGH receptors in bovine and rat tissues were not successful until the number of atoms of [^{125}I] per bGH molecule was decreased to compensate for the fewer tyrosine residues in bGH as compared with hGH. After this modification, bGH bound specifically to rat and bovine liver and rat kidney membranes with high affinity. This demonstration of specific bGH binding to rat liver membranes is in contrast to previous studies which failed to demonstrate specific binding (Herington et al., 1976; Posner, 1976). The lack of PRL competition with the GH binding sites in bovine liver membranes supports the contention that bGH binds to specific somatotrophic sites (Posner, 1976). The minimal displacement of bGH by PRL at very high concentrations may have been due to slight contamination of prolactin with GH.

The reason bovine kidney membranes did not bind bGH even though insulin was bound is not readily apparent. In both rats and sheep, the kidney has been shown to be important in GH clearance from plasma (Wallace et al., 1972,; Wallace and Stacy, 1975). GH is also concentrated in the rat kidney (Retegni-Sardou et al., 1977; Johnson and Maack, 1977). In the case of sheep, the kidney is necessary for GH to exert its lipolytic effect (Wallace et al., 1970). The explanation may be in the basic response differences of rats and ruminants to GH. In rats, GH is responsible for production of somatomedin, while in lambs, GH was not shown to increase somatomedin production (Falconer et al., 1977).

REFERENCES

Anderson, O., J. Gliemann, and S. Gammeltoft. 1977. Receptor binding and biological effects of insulin in human adipocytes. Diabetologia 13: 589-593.

Barr, A. J., J. H. Goodnight, J. P. Sall, W. H. Blair, and D. M. Chilko. 1979. SAS User's Guide SAS Institute, Inc., Raleigh, N.C.

Boyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. Scand. J. Clin. Lab. Invest. (Suppl 97) 21: 88-89.

Cole, R. D., and C. H. Li. 1955. Studies on pituitary lactogenic hormone; a simplified procedure of isolation. J. Biol. Chem. 213: 197-201.

Cuatrecasas, P. 1971. Properties of the insulin receptor of isolated fat cell membrane. J. Biol. Chem. 246: 7265-7274.

De Meyts, P. 1976a. Insulin and growth hormone receptors in human cultured lymphocytes and peripheral blood monocytes. Page 301 in M. Blecher, ed., Methods in Molecular Biology: Methods in Receptor Research, Part 1. Marcel Dekker, Inc., New York.

De Meyts, P. 1976b. The negative cooperativity of insulin receptors: a model for the regulation of hormone recognition by target cells. Pages 17-31 in R.F. Baers and E. G. Gassett, eds., Cell Membrane Receptors for Viruses, Antigens and Antibodies, Polypeptide Hormones and Small Molecules. Raven Press, New York.

Falconer, J., H. J. Buttle, and J. M. Forbes. 1977. Somatomedin levels in the blood of hypophysectomized sheep given growth hormone or prolactin. J. Endocrinol. 72: 30-31, 1977.

Feldman, H. A. 1972. Mathematical theory of complex ligand-binding systems at equilibrium: some methods of parameter fitting. Ann. Biochem. 48: 317-338.

Gavin, J. R., J. Roth, P. Jen, and P. Freychet. 1972. Insulin receptors in human circulating cells and fibroblasts. Proc. Natl. Acad. Sci. USA 69: 747-751.

Gavin, J. R., P. Gordon, J. Roth, J. A. Archer, and D. N. Buell. 1973. Characteristics of the human lymphocyte insulin receptor. J. Biol. Chem. 248:2202-2207.

Gill, R. D., and I. C. Hart. 1980. Properties of insulin and glucagon receptors on sheep hepatocytes: A comparison of hormone binding and plasma hormone and metabolites in lactating and non-lactating ewes. J. Endocrinol. 84: 237-247.

Ginsberg, B. H., C. R. Kahn, and J. Roch. 1976. The insulin receptor of the turkey erythrocyte characterization of the membrane-bound receptor. Biochim. Biophys. Acta 443: 222-242.

Herington, A. C., L. S. Phillips, and W. H. Daughaday. 1976. Pituitary regulation of human growth hormone binding sites in rat liver membranes. Metabolism 25: 341-353.

Hinterberger, W., K. Kletter, and W. Waldhausl. 1976. Binding of human growth hormone to human peripheral lymphocytes. Horm. Metab. Res. 8: 492-493.

Hollenberg, M. D., and P. Cuatrecasas. 1976. Methods for the biochemical identification of insulin receptors. Page 450 in M. Blecher, ed., Methods in Molecular Biology: Methods in Receptor Research, Part 2, Marcel Dekker, Inc., New York.

Johnson, V., and T. Maack. 1977. Renal extraction, filtration, absorption and catabolism of growth hormone. Am. J. Physiol. 233: F185-F196.

Kahn, C. R., and J. Roth. 1976. Insulin receptors in disease states. Page 15 in G.W. Levey, ed., Hormone-Receptor Interaction: Molecular Aspects, Marcel-Dekker, Inc., New York.

Kahn, C. R., I. D. Goldfine, D. M. Neville, Jr., and P. De Meyts. 1978. Alterations in insulin binding induced by changes in vivo in the levels of glucocorticoids and growth hormone. Endocrinology 103: 1054-1066.

Kemmler, W., R. Renner, A. Zynamon, and K. D. Hep. 1978. Interactions between insulins and liver membrane receptors of guinea pig, calf and chicken; exclusion of a species-specific insulin receptor. Biochim. Biophys. Acta 543: 349-356.

Kono, T., and F. W. Barham. 1971. The relationship between the insulin-binding capacity of fat cells and the cellular response to insulin. J. Biol. Chem. 246:6210-6216.

Li, C.H. 1954. A simplified procedure for the isolation of hypophyseal growth hormone. J. Biol. Chem. 211: 555-558.

Lowry, O. H., N.J. Rosenbrough, A.l. Rarr, and R.J. Randall. 1951. Protein measurement with the Folin reagent. J. Biol. Chem. 193: 265-275.

Muggeo, M., B. H. Ginsberg, J. Roth, D. Neville, P. De Meyts, and R. Kahn. 1979. The insulin receptor in vertebrates is functionally more conserved during evolution than insulin itself. Endocrinology 140: 1393-1402.

Norby, J. G., P. Ottolenghi, and J. Hensen. 1980. Scatchard plot: Common misinterpretation of binding experiments. Anal. Biochem. 102: 318-320.

Olefsky, J. M. 1976. Effects of fasting on insulin binding, glucose transport, and glucose oxidation in isolated rat adipocytes. J. Clin. Invest. 58: 1450-1460.

Olefsky, J., V. C. Bacon, and S. Baun. 1976. Insulin receptors of skeletal muscle: Specific insulin binding sites and demonstration of decreased numbers of sites in obese rats. Metabolism 25: 179-191.

Olefsky, J. M., and H. Chang. 1978. Insulin binding to adipocytes: Evidence for functionally distinct receptors. Diabetes 27: 946-958.

Papkoff, H., C. H. Li, and W. Liu. 1962. The isolation and characterization of growth hormone from porcine pituitaries. Arch. Biochem. Biophys. 96: 216-225.

Posner, B. I. 1976. Characterization and modulation of growth hormone and prolactin binding in mouse liver. Endocrinology 98: 645-654.

Retegni-Sardou, L.A., L. A. Scaramal, J. M. Dellacha, and A.C. Paladini. 1977. Rates of concentration and digestion of radioactive growth hormone preparations injected in rats, as measured by the amount and nature of radioactivity in the tissues. Mol. Cell. Biochem. 16: 87-96.

Rosen, P., B. Ehrich, E. Junger, H. F. Bubenzerard, and L. Kuhn. 1979. Binding and degradation of insulin by plasma membranes from bovine liver isolated by a large scale preparation. Biochem. Biophys. Acta 587:593-605.

Rosen, P., M. Simon, H. Reinauer, H. J. Friesen, C. Diaconescu, and D. Brandenburg. 1980. Binding of insulins to bovine liver plasma membranes. Use of insulin analogues modified at the A1 Residue. Biochem. J. 186: 945-952.

Scatchard, G. 1949. The attraction of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. 51: 660-673.

Takano, K., K. Hall, L. Fryklund, and H. Sivertsson. 1976. Binding of somatomedins and insulin to plasma membranes from rat and monkey tissue. Horm. Metab. Res. 8: 16-24.

Wallace, A. L. C., and K. A. Ferguson. 1963. The preparation of sheep growth hormone. J. Endocrinol. 26: 259-265.

Wallace, A. L. C., B. D. Stacy, and G. D. Thornburn. 1970. The effect of kidney ligation on the release of plasma free fatty acids following the injection of growth hormone. J. Endocrinol. 48: 297-298.

Wallace, A. L. C., B. D. Stacy, and G. D. Thornburn. 1972. The fate of radioiodinated sheep-growth hormone in intact and nephrectomized sheep. Pflugers Archiv. 31: 25-37.

Wallace, A. L. C., and B. D. Stacy. 1975. Disappearance of ^{125}I-labeled rat growth hormone in nephrectomized and sham operated rats. Horm. Metab. Res. 7: 135-138.

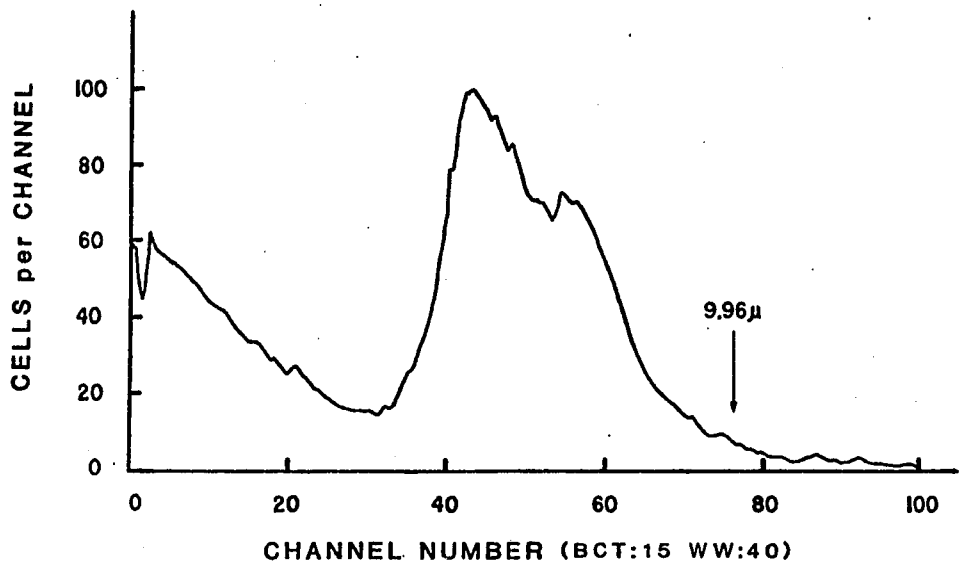

Figure 1: Mononuclear blood cell size profile of Ficoll-Hypaque gradient isolates; Coulter cell channelizer was set at a base cell setting of 15% and window width setting of 40%. The major peak (channel 35-50) contained primarily small lymphocytes ($\approx 6\mu$) while the smaller secondary peak 50-65, contained large "monocytic" cells. The large cells accounted for 20-35% of the total mononuclear cells present in bovine cell preparations. Platelets and red cells were contained in channels 0-30.

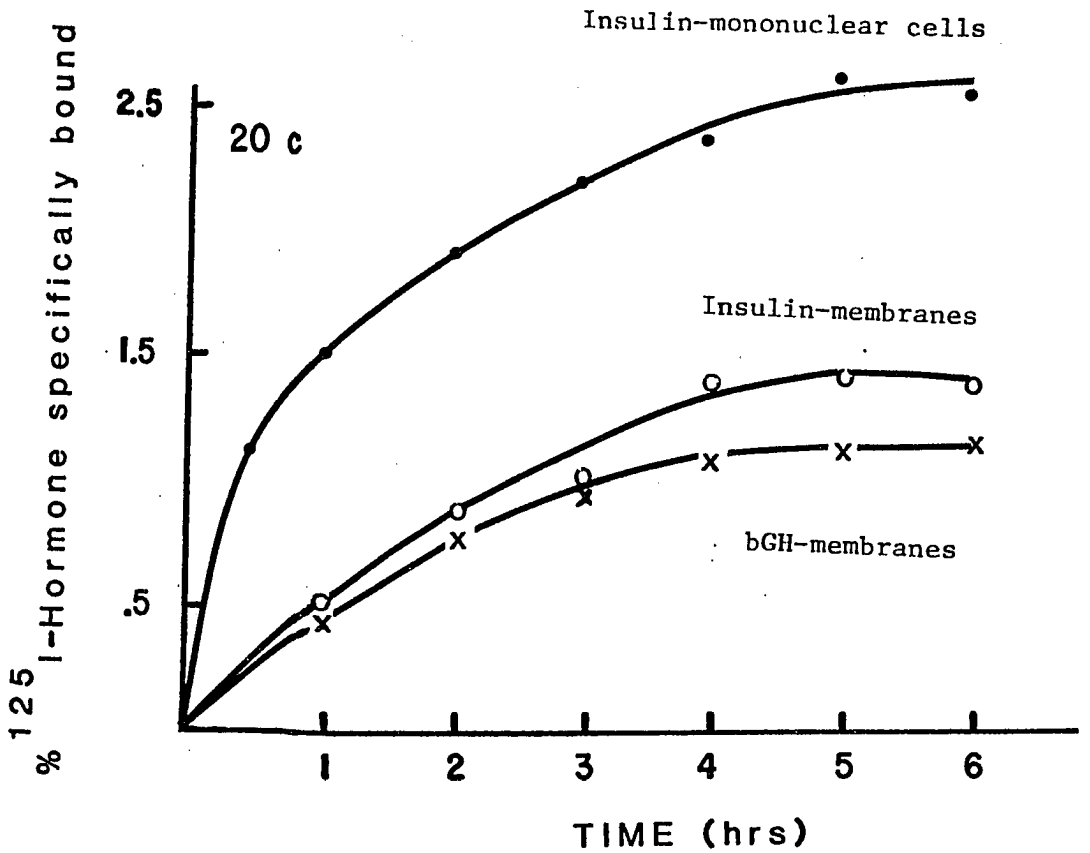

Figure 2: Time course of [^{125}I] bovine insulin and [^{125}I] bovine growth hormone binding to bovine liver plasma membranes (binding per 200 μg protein) and [^{125}I] insulin to bovine mononuclear cells (25 x 10^6 cells). Nonspecific binding (binding in presence of 10^5 ng/ml cold insulin) is subtracted from each time point.

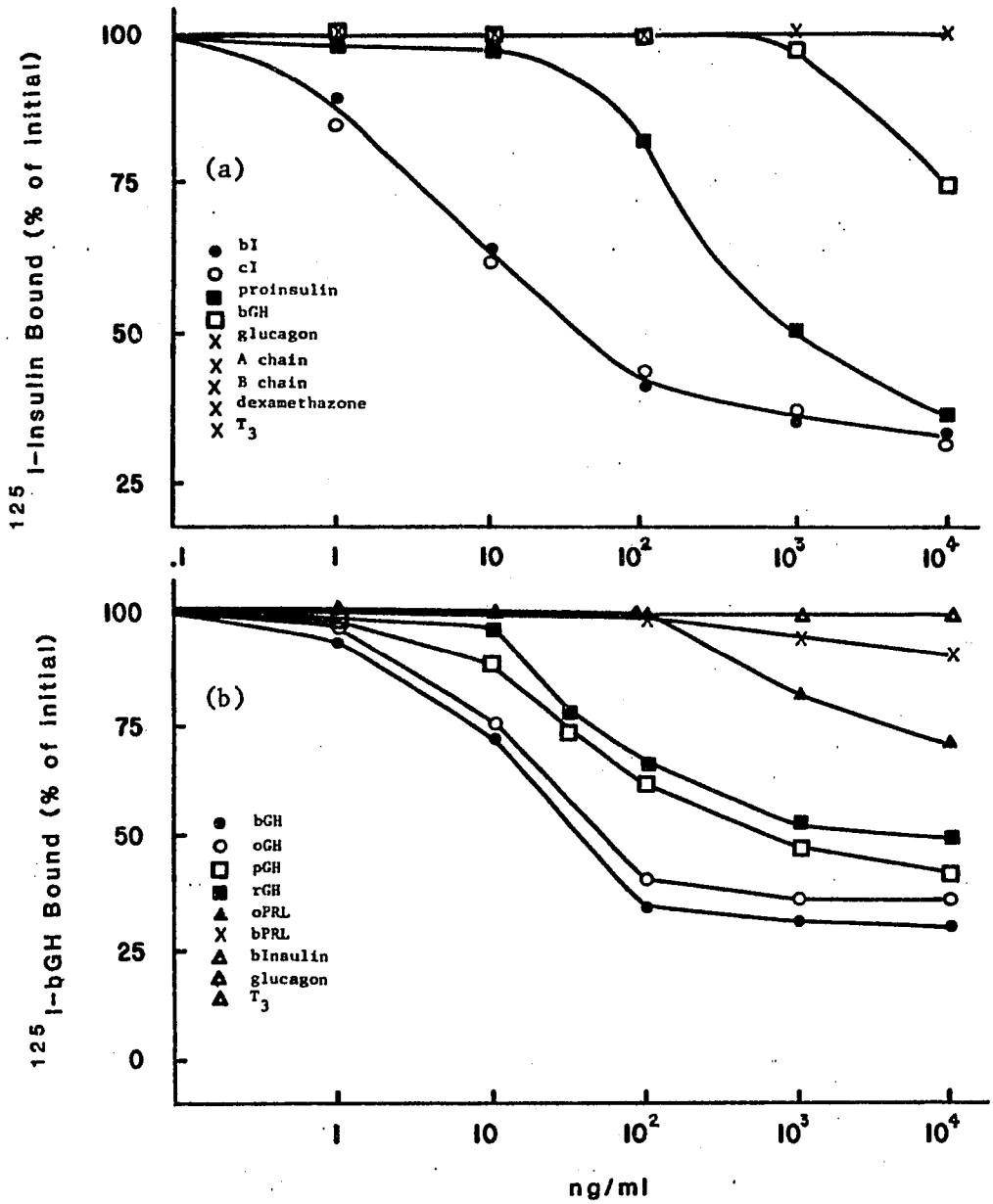

Figure 3: Competition of various hormones for bovine insulin (panel a) and bovine growth hormone (panel b) receptor sites in bovine liver plasma membranes (binding per 200 μg protein). Membranes were incubated for 6 hrs at 20 C with increasing concentrations of unlabeled hormones. Results are expressed as a percentage of the [^{125}I]-labeled hormone specifically bound when no cold hormone was added.

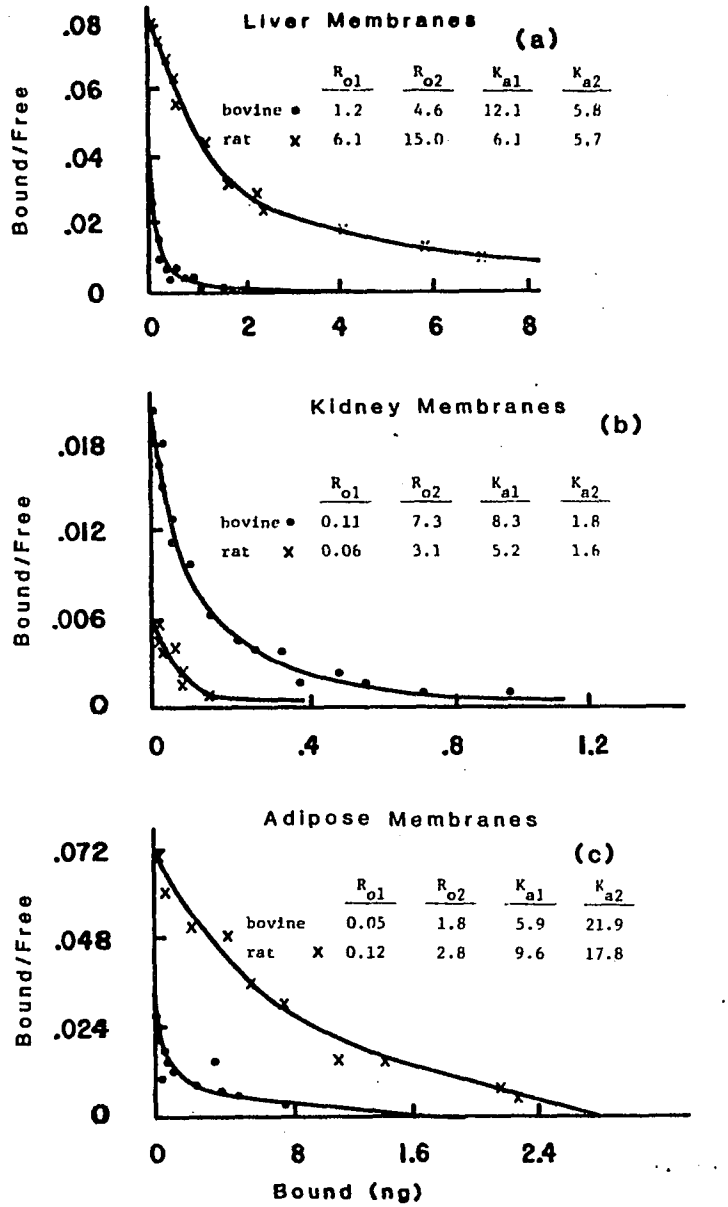

Figure 4: Comparison of [^{125}I] bovine insulin binding to plasma membranes (200 μg protein) from rat and bovine liver (panel a), kidney (panel b), and adipose (panel c). Two class Scatchard binding parameters, determined by a least squares iterative procedure, are shown for each tissue. Ro values are in ng of insulin per 200 μg of protein while Ka_1 and Ka_2 values are x 10^8 M^{-1} and x 10^7 M^{-1} respectively. Solid line represents prediction equation derived from the least squares fit of the data (see Materials and Methods).

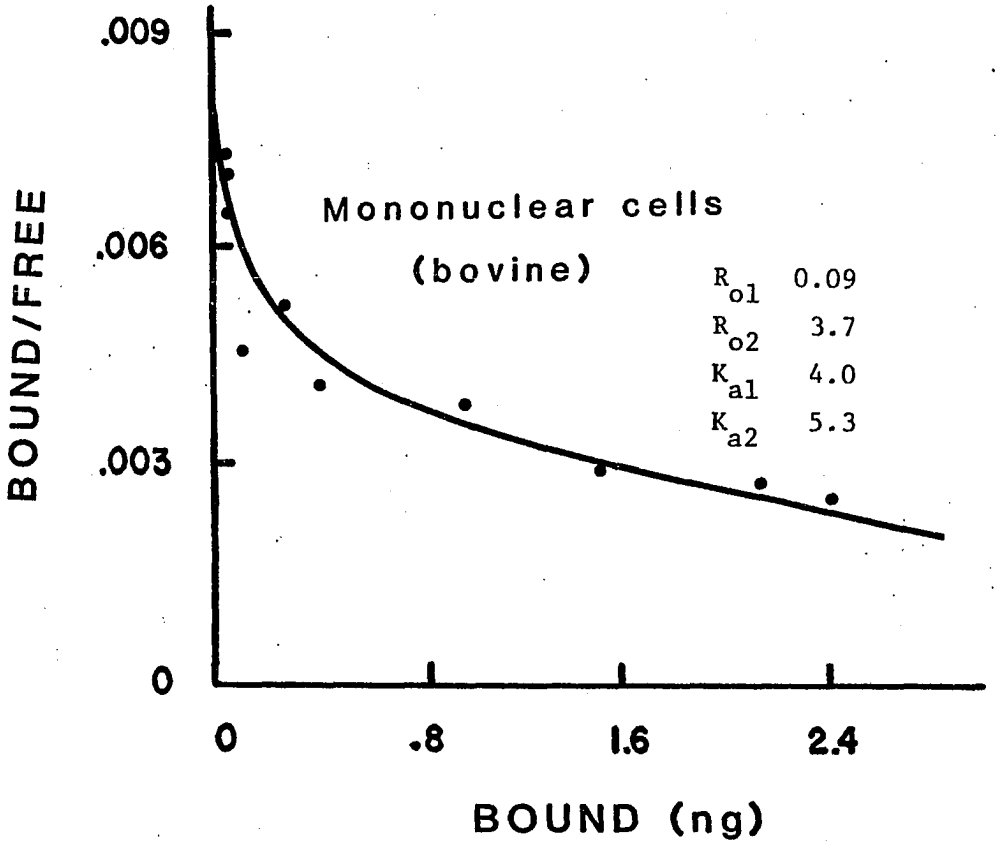

Figure 5: Binding of [^{125}I] bovine insulin to bovine mononuclear cells (25 x 10^6 cells) Scatchard binding parameters were estimated for a two class receptor model by iterative least squares procedure. (Ro values are in ng, while Ka_1 and Ka_2 values are x 10^8 M^{-1} and 10^7 M^{-1}, respectively).

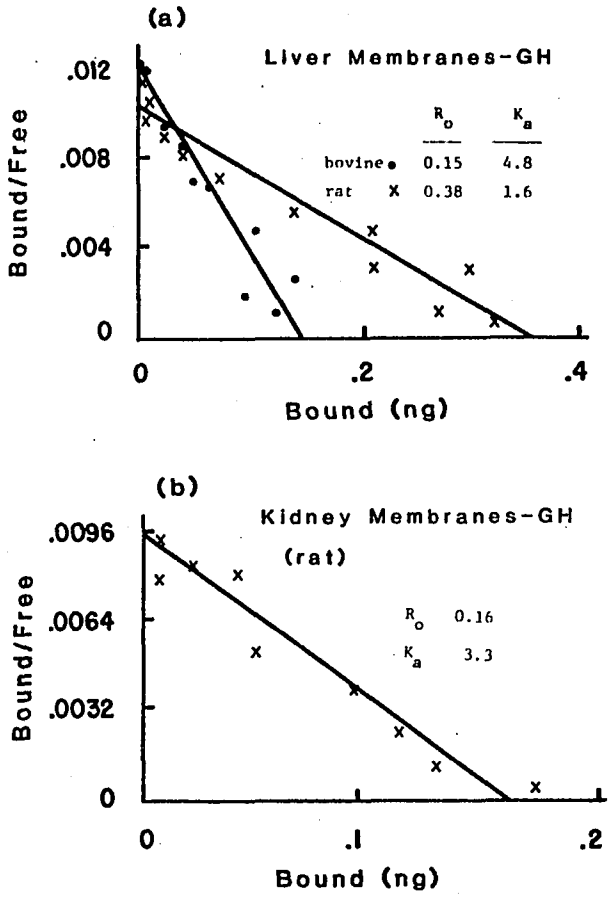

Figure 6: [^{125}I] bovine growth hormone binding to isolated membranes (200μg) from liver (panel a) and kidney (panel b) of bovine and rat. Binding parameters for one class of receptors determined by Scatchard analysis are shown. Ro values are in ng of hormone/200μg of protein while Ka values are x 10^8 M^{-1}.

SECTION II: INSULIN AND GROWTH HORMONE RECEPTORS IN CATTLE TISSUES: COMPARISON OF BINDING IN CATTLE OF LARGE AND SMALL FRAME SIZE

Steven Nissen and Allen Trenkle

Department of Animal Science

Iowa State University

Ames, Iowa 50011

ABSTRACT

Binding of bovine insulin and bovine growth hormone (GH) was characterized in tissues from large and small framed cattle to determine if receptor modulation plays a role in regulating hormone action. Mononuclear cells from small framed cattle had lower binding capacity than did large framed cattle, but this difference was small compared with the increased concentrations of plasma insulin observed in small cattle. Insulin receptor changes were not reflected equally in blood mononuclear cells and membranes of liver and kidney. There was no difference in GH binding to liver membranes of large and small framed cattle. These studies suggest that plasma hormone concentrations adequately reflect the endocrine status of cattle under conditions of moderate changes in insulin and GH concentrations.

INTRODUCTION

Recent knowledge of specific hormone receptors has emphasized the importance of determining concentrations of hormones in body fluids as well as binding of hormones to the target tissues. In nonruminants, it appears that changes in plasma insulin concentrations are countered by opposite changes in insulin binding (Kahn et al., 1973, Olefsky, 1976). This counter regulation between insulin and its receptor necessitates measurement of both hormone concentration and hormone binding to estimate the *in vivo* effects of the hormone. It is unknown whether the changes in plasma insulin and GH that accompany changes in cattle growth (Joakimson and Blom, 1976, Trenkle and Topel, 1978) and metabolism (Hart et al., 1978) are countered by opposite changes in receptor binding. Until insulin and growth hormone receptors are measured in cattle tissues, this plasma hormone data will have limited application.

In the experiments reported here, insulin and growth hormone receptors were measured in various tissues from cattle of large and small frame size. The purpose of this study was to determine if combining the hormone receptor data with plasma hormone concentrations would more fully explain the importance of insulin and GH in regulating growth of cattle.

MATERIALS AND METHODS

Animals

Samples were taken from large and small framed cattle being studied in four separate experiments. In the first two studies, blood was taken for mononuclear cell isolation while in the latter two studies tissue samples were also taken for isolation of plasma membranes. In all experiments, animals were fed ad libitum prior to sampling. Diets were generally categorized as medium to high energy of which 50 to 85% of the diet consisted of corn grain. Adequate minerals, vitamins and protein were added to all diets. The Simmental and Limousin cattle representing large frame size were at least 3/4 purebred. Purebred Aberden Angus (Angus) or Angus x Hereford cattle represented small frame size. The Holstein cattle sampled in one experiment were purebred.

Experiment 1. Angus, Simmental and Holstein (5 each) were selected at weaning, accustomed to handling with halters and fed a diet of which corn grain made up 80% of the dry matter. Animal weights and ages at the time of the experiment are included in Table 1. A 250 ml blood sample was obtained by jugular catheter for isolation of mononuclear cells.

Experiment 2. Blood was taken from Angus and Simmental cattle being fed a high energy diet (80% corn grain). Ages and carcass weights are included in Table 2. Animals were restrained in a catch chute and blood obtained by jugular vein puncture. Angus animals were slaughtered the day after blood sampling and Simmental were slaughtered 45 days later.

Experiment 3. The cattle in this experiment were grouped as large or small frame size based on weight at weaning (45 days). The breed composition of the large animals was predominantly Simmental while that of the small animals was primarily Angus. Animals of both groups were fed a high energy diet (85% corn grain). A week before slaughter, a catheter was placed in the jugular vein and the following day blood samples taken every 30 minutes for six hours. These plasma samples were then pooled for hormone assay. Blood was collected at the time of slaughter for isolation of mononuclear cells and samples of kidney, liver and mesenteric fat were taken for isolation of plasma membranes.

Experiment 4. Blood and liver samples were taken from Limousin, Angus and Simmental steers fed a medium energy diet (65% corn grain), weights and ages are given in Table 4. The day prior to slaughter, jugular blood was collected by venipuncture. Liver samples were obtained at slaughter.

Cell and membrane isolation

Mononuclear cells were isolated by a modification of the Ficoll-Hypaque density gradient method (DeMeyts, 1976, Nissen and Trenkle, 1981). Plasma membranes were isolated by the centrifugation procedure outlined by Hollenberg and Cuatrecasas (1976).

Assay procedures

Bovine [^{125}I]insulin and [^{125}I]growth hormone (bGH) binding to circulating mononuclear cells and plasma membranes are described in the previous paper (Nissen and Trenkle, 1981). Insulin was assayed by the

procedure outlined by Trenkle, 1972. Growth hormone radioimmunoassay was carried out by the procedure of Trenkle, 1976. Binding results are reported here on the basis of a two class Scatchard binding model (Nissen and Trenkle, 1981).

Determining the amount of insulin or GH bound in vivo was estimated from plasma hormone concentration and in vitro binding data. A curve was constructed from the in vitro binding data of each animal relating the concentration of hormone (0 to 10 ng/ml) to the amount of hormone bound (ng per 25 x 10^6 mononuclear cells and ng per 200 µg membrane protein). Substitution of plasma hormone concentration into the equation (quadratic regression) gave an estimate of in vivo binding. The assumptions were that plasma hormone concentrations were similar to those bathing tissues and that the in vitro binding estimates were representative of the events occurring in vivo.

Comparison of binding parameters and hormone concentrations between cattle types was carried out using analysis of variance. Simple and partial correlations were done according to Steel and Torrie (1960). All possible correlations were calculated between insulin and GH binding parameters.

RESULTS

Experiment 1. Results of insulin binding to mononuclear cells are presented in Table 1. A major difference observed in this experiment was the increase in maximum insulin binding by Holstein mononuclear cells from Holstein compared with those from Angus or Simmental. This difference was due primarily to higher receptor numbers (Ro_1 and Ro_2). Taking into account the plasma insulin concentrations, the Holstein animals had almost twice the estimated *in vivo* binding of insulin as compared with Angus or Simmental. Plasma concentrations of GH were higher in Simmental and Holstein than in Angus. Growth hormone did not specifically bind to bovine mononuclear cells (Nissen and Trenkle, 1981).

Experiment 2. Insulin binding to mononuclear cells of Angus and Simmental cattle is presented in Table 2. The maximum binding percentage was significantly lower in Angus compared with Simmental due primarily to a large decrease in low affinity receptor. The high affinity binding constant for insulin was slightly higher in Angus. Plasma insulin was not measured in these animals.

Experiment 3. The results of experiment 3 are presented in Table 3. Plasma insulin concentrations in the small animals were much higher than in the large animals yet the only Scatchard binding parameter significantly different was a small decrease in the number of high affinity receptors on the mononuclear cells from the small animals. Small animals also had a large decrease (not significant) in maximum insulin binding to adipose membranes. Estimates of *in vivo* binding indicated the small animals

had more insulin bound to liver and kidney membranes than did large type cattle, primarily because of elevated insulin concentrations. The only exception to this may be in adipose membranes, but because of limited material binding parameters could not be estimated.

The amount of GH bound to liver membranes and plasma GH concentrations was not different in the two types of cattle.

Experiment 4. The results of experiment 4 are summarized in Table 4. Because of the limited number of animals used in this experiment, few binding parameters approached significance. The results, however, generally support trends seen in the previous three experiments, namely a tendency toward increased receptor numbers (R_{o1} and R_{o2}) and decreased affinity (K_{a1} and K_{a2}) in the larger leaner breeds. Maximum binding of GH tended to be higher in Simmental and Limousin animals. Coupled with high plasma GH, the Simmental tended to have higher estimates of membrane bound GH.

Some of the correlations between binding parameters (across all experiments) are presented in Table 5. Simple correlations indicate a constant negative relationship between receptor numbers and affinity constants for both insulin and GH. Removing effects of breed and experiment (partial r) does little to affect these relationships. There appears to be no correlation between concentrations of insulin in plasma and any insulin binding parameters in any of the tissues studied (all correlations $<$ .20; data not shown). Maximum binding of GH also appears to be related to insulin binding to the monocyte high affinity receptor number (R_{o1}), while plasma insulin is negatively correlated with GH R_o (Table 5).

DISCUSSION

Decreases in insulin binding have been reported to occur in obese mice and humans in the presence of increased plasma insulin concentrations (Kahn et al., 1973, Olefsky, 1976). In studies reported here, the insulin binding capacity in smaller framed cattle is decreased but this decrease is not in proportion to the higher concentration of insulin in these animals. Thus, the increase in insulin concentration noted here in small breeds of cattle, in addition to the increased concentrations that occur in cattle with age (Trenkle and Topel, 1978), probably result in more insulin binding to tissues. Postreceptor considerations notwithstanding, the increased insulin binding should bring about a greater metabolic effect of insulin in the tissues of these small framed animals.

Like the insulin receptor, GH receptors changed little in response to changes in plasma GH concentrations. If GH receptors in cattle are unchanged in response to elevations in plasma GH concentrations, the decrease in plasma GH seen with aging (Joakimsen and Blom, 1976, Trenkle and Topel, 1978) and the decrease in plasma GH seen in fat cattle (Trenkle and Topel, 1978) probably result in a decrease in the amount of GH bound to the tissue. This should theoretically result in a diminished effect of the hormone on the tissue.

In humans, insulin binding to mononuclear cells and adipocytes are considered to be related (Olefsky, 1976) but extensive studies have not been conducted. The results reported here indicate little parallelism between insulin binding to mononuclear cells and binding to liver or kidney membranes.

Direct measurement of insulin binding in adipose tissue and GH binding to muscle would be useful. Unfortunately, isolation of bovine fat or muscle cells (Olefsky et al, 1976) is difficult. The enzymes necessary to free individual cells from connective tissues probably would alter surface binding sites (Kono and Barham, 1971). The results of trial 3 in which insulin binding to adipose membranes was measured (Table 3) indicate a large decrease in insulin binding in the small animals. Insufficient material was available to more fully characterize the binding in each animal. In the previous paper (Nissen and Trenkle, 1981), binding characterization of membranes isolated from adipose tissue indicated the presence of high affinity insulin receptors. Attempts to isolate skeletal muscle membranes by the method described here were unsuccessful.

The high negative correlation between both insulin and GH affinity and receptor numbers noted here (Table 5) is also evident in several other studies reporting changes in receptor binding (Herrington et al., 1976, Kahn et al., 1973, Kahn et al., 1978). This may be a result of inaccurate estimates of Scatchard binding parameters or may be an event related to receptor "down regulation". As cell surface receptors increase (R_o) and are in turn closer together, the affinity (K_a) may be decreased. Thus, a type of modulation of hormone action by the receptors themselves may take place on the cell surface. This may be a form of or an adjunct to the hormone mediated "down regulation" that occurs in response to changes in hormone concentration as reported by Olefsky (1976).

The results obtained with the tissues studied in these experiments are consistent with the concept that receptors for insulin and GH play a secondary role to plasma hormone concentration in regulating the action of insulin and GH in bovine tissues. In effect, these receptors do not appear to be fully "down regulated" by plasma hormone concentrations. Additionally, it appears that interaction of surface receptors themselves may mediate hormone binding and may be more important than hormone concentrations in controlling receptor binding within the narrow physiological range of hormone concentrations.

REFERENCES

DeMeyts, P. 1976. Insulin and growth hormone receptors in human cultural lymphocytes and peripheral blood monocytes. Page 301 in M. Blecher, ed. Methods in Molecular Biology: Methods in Receptor Research, Part 1. Marcel Dekker, Inc., New York.

Hart, I. C., J. A. Bines, S. V. Morant, and J. L. Ridley. 1978. Endocrine control of energy metabolism in the cow: comparison of the levels of hormone (prolactin, growth hormone, insulin and thyroxine) and metabolites in the plasma of high- and low-yielding cattle at various stages of lactation. J. Endocrinol. 77: 333-345.

Herrington, A. C., L. S. Phillips, and W. H. Daughaday. 1976. Pituitary regulation of human growth hormone binding sites in rat liver membranes. Metabolism 25: 341-353.

Hollenberg, M. D., and P. Cuatrecasas. 1976. Methods for the biochemical identification of insulin receptors. Page 450 in M. Blecher, ed. Methods in Molecular Biology: Methods in Receptor Research, Part 2. Marcel Dekker, Inc., New York.

Joakimsen, O., and A. K. Blom. 1976. Growth hormone concentration in jugular blood plasma in relation to growth rate and age in young bulls. Acta Agri. Scand. 26: 239-242.

Kahn, C. R., D. M. Neville, and J. Roth. 1973. Insulin-receptor interaction in the obese-hyperglycemic mouse. A model of insulin resistance. J. Biol. Chem. 248: 244-250.

Kahn, C. R., I. D. Goldfine, D. M. Neville, and P. DeMeyts. 1978. Alterations in insulin binding induced by changes in vivo in the levels of glucocorticoids and growth hormone. Endocrinology 103: 1054-1065.

Kono, T., and F. W. Barham. 1971. The relationship between the insulin-binding capacity of fat cells and the cellular response to insulin. J. Biol. Chem. 246: 6210-6216.

Nissen, S., and A. Trenkle. 1981. Insulin and bovine growth hormone receptors in cattle tissues; characterization and comparison with rat tissues. Horm. Metab. Res. (submitted).

Olefsky, J. M. 1976. Decreased insulin binding to adipocytes and circulating mormocytes from obese subjects. J. Clin. Invest. 57: 1165-1172.

Olefsky, J., V. C. Bacon, and S. Baun. 1976. Insulin receptors of skeletal muscle: Specific insulin binding sites and demonstration of decreased numbers of sites in obese rats. Metabolism 25: 179-191.

Steel, R. G. D., and J. H. Torrie. 1960. Principles and Procedures of Statistics. McGraw-Hill, New York.

Trenkle, A. 1972. Radioimmunoassay of plasma hormones: Review of plasma insulin in ruminants. J. Dairy Sci. 55: 1200-1206.

Trenkle, A. 1976. Estimates of kinetic parameters of growth hormone metabolism in fed and fasted calves and sheep. J. Anim. Sci. 54: 1035-1040.

Trenkle, A., and D. G. Topel. 1978. Relationships of some endocrine measurements to growth and carcass composition of cattle. J. Anim. Sci. 46: 1604-1609.

Table 1. Insulin binding characteristics of mononuclear cells of Angus, Simmental and Holstein cattle

	Angus	Simmental	Holstein	Pr > F[a]
n	5	5	5	--
Age (mo)	10	10	7	--
Weight (kg)	298	300	289	--
Plasma hormones				
Insulin (ng/ml)	2.22	1.88	2.71	.15
GH (ng/ml)	3.70	13.70	10.46	.08
Scatchard binding analysis[b]				
Ro_1 (ng)	.040	.039	.066*	.06
Ro_2 (ng)	1.25	1.07	2.62*	.01
Ka_1 ($M^{-1} \times 10^8$)	8.93	7.01	6.90	.53
Ka_2 ($M^{-1} \times 10^7$)	1.52	1.47	1.01	.92
Max binding %[c]	.684	.667	1.13*	.01
Cell Bound (ng)[d]	.011	.012	.020*	.01

[a]Overall analysis of variance significance. Significant difference ($p < .05$) from other groups is indicated by*.

[b]Scatchard analysis on the basis of two independent classes of receptors (25×10^6 mononuclear cells).

[c]Maximum binding percent is the percent of ^{125}I-insulin added that is bound to 25×10^6 cells when no cold insulin is added. Nonspecific binding (binding in presence of 10^5 ng/ml cold insulin) is subtrated from this value.

[d]Cell bound insulin is the calculated amount of insulin that should be bound to 25×10^6 cells *in vivo* at the concentration of insulin measured in plasma.

Table 2. Effect of breed on insulin binding to mononuclear blood cells[a]

	Angus	Simmental	Breed Pr > F
n	20	15	--
Age (mo)	15	10	--
Carcass weight (kg)	401	288	.001
Ro1 (ng)	.014	.038	.62
Ro2 (ng)	.763	1.20	.01
Ka1 ($M^{-1} \times 10^8$)	12.00	9.49	.01
Ka2 ($M^{-1} \times 10^7$)	1.14	1.56	.10
Max binding %	.27	.65	.05

[a]See Table 1 for further explanation of table.

Table 3. Insulin and growth hormone binding parameters in cattle selected at weaning for large and small framed types[a]

	Parameter	Small	Large	Pr > F
	n	7	7	
	Age (mo)	13	13	
	Body weight (kg)	352	429	.01
	Carcass weight (kg)	205	265	.02
Plasma hormones	Insulin (ng/ml)	2.92	1.33	.01
	Growth hormone (ng/ml)	3.70	3.71	.90
Insulin binding				
Mononuclear cells	Ro1 (ng)	.071	.100	.05
	Ro2 (ng)	5.50	6.11	.77
	Ka1 ($M^{-1} \times 10^8$)	5.5	5.2	.72
	Ka2 ($M^{-1} \times 10^7$)	.70	.72	.89
	Max binding %	1.35	1.61	.41
	Cell bound (ng)	.033	.025	.49
Liver membranes[b]	Ro1 (ng)	.119	.102	.36
	Ro2 (ng)	1.62	1.68	.36
	Ka1 ($M^{-1} \times 10^8$)	12.3	12.7	.88
	Ka2 ($M^{-1} \times 10^7$)	.76	1.06	.49
	Max binding %	2.38	2.30	.76
	Membrane bound (ng)	.038	.020	.01
Kidney membranes	Ro1 (ng)	.120	.136	.67
	Ro2 (ng)	1.08	1.34	.51
	Ka1 ($M^{-1} \times 10^8$)	10.2	14.5	.62
	Ka2 ($M^{-1} \times 10^7$)	1.4	1.0	.65
	Max bindng %	1.85	1.71	.48
	Membrane bound (ng)	.037	.018	.03
Adipose membranes[c]	Max binding %	.205	.988	.08
Gowth hormone binding				
Liver membranes	Ro (ng)	.118	.180	.26
	Ka ($M^{-1} \times 10^8$)	7.9	5.5	.24
	Max binding %	1.19	1.17	.93
	Membrane bound (ng)	.027	.030	.74

[a]See Table 1 footnotes for explanation of table.

[b]All membrane binding presented on the basis of 200 μg of protein.

[c]Insufficient fat membrane available for Scatchard analysis.

Table 4. Insulin and growth hormone binding to Angus, Limousin and Simmental cattle[a]

	Parameter	Angus	Limousin	Simmental	Pr > F
	n	4	4	3	---
	Carcass weight (kg)	315	389	396	---
	Age (mo)	25	22	22	---
Plasma hormones	Insulin (ng/ml)	1.90	1.34	1.30	.10
	Growth hormone (ng/ml)	3.11	3.10	5.04	.05
Insulin binding					
Mononuclear cells	Ro1 (ng)	.083	.066	.172	.62
	Ro2 (ng)	2.16	3.09	5.21	.47
	Ka1 ($M^{-1} \times 10^8$)	6.9	6.4	1.86	.02
	Ka2 ($M^{-1} \times 10^7$)	1.5	1.2	.73	.25
	Max binding %	1.36	1.22	1.02	.52
	Cell bound (ng)	.021	0.16	.013	.33
Liver membranes	Ro1 (ng)	.125	.130	.155	.61
	Ro2	1.58	1.41	1.40	.90
	Ka1 ($M^{-1} \times 10^8$)	13.0	13.1	11.6	.63
	Ka2 ($M^{-1} \times 10^7$)	.64	.72	.63	.97
	Max binding %	2.82	2.91	3.18	.82
	Membrane bound (ng)	.030	.025	.028	.66
Growth hormone binding					
Liver membranes	Ro (ng)	.352	.380	.241	.77
	Ka ($M^{-1} \times 10^8$)	4.71	4.71	8.16	.18
	Max binding %	2.44	2.67	2.88	.17
	Membrane bound (ng)	.050	.057	.082	.18

[a]See Table 1 for further explanation of Table 4.

Table 5. Correlations between affinity constants and receptor numbers (all experiments)

		n	Simple r	Partial[a] r
Insulin				
Mononuclear cells	Rol vs Kal	87	-.49	-.28
	Ro2 vs Ka2	87	-.31	-.24
Liver membranes	Rol VS Kal	25	-.62	-.65
	Ro2 vs Ka2	25	-.21	-.32
GH				
Liver membranes	Ro vs Ka	25	-.71	-.80
GH-Insulin relationship				
Plasma insulin vs GH Ro		25	--	-.46

[a]Removing effects of breed and experiment

GENERAL DISCUSSION

The studies reported here suggest insulin and GH receptors are not the primary regulators of insulin and GH action. The changes in receptor binding were usually small compared to the much larger changes in plasma hormone concentrations, the net result being that small cattle probably have less GH and more insulin bound to their tissues than do large lean animals. However, these differences are subject to postreceptor modulation which could change the ultimate effect of the hormone. Postreceptor events were not measured in these studies. Future studies will necessitate such measurements if further inferences into hormone physiology and growth are to be made.

The basis for any conclusions concerning changes in hormone receptors is the validity of the *in vitro* binding assay. In general, all peptide binding techniques suffer on several fronts. Among the worst are nonphysiological binding conditions, ambiguity in data analysis and absolute variation in binding measurements. All these cloud the already difficult transition between *in vitro* and *in vivo* binding.

The fact that hormone binding is optimized for maximum binding usually results in nonphysiological pH, temperature and ionic environment. Thus, binding is maximized but other processes such as degradation and endocytosis are minimized. The question is if this optimized *in vitro* system can be translated into the *in vivo* situation where several processes are occurring simultaneously. It would seem hormone responsiveness or sensitivity should be measured as an integrated cellular process rather than isolating the parts of the system.

Assay variation also is important in interpreting the binding results and is composed of at least two components. First, the variation in bound and free separation due to cell breakage, inadequate mixing or inaccurate pipeting which necessitates triplicate analysis at each binding point. Secondly, the Scatchard analysis of binding data is subject to tremendous subjectivity. The least squares method of estimating Scatchard binding parameters used here eliminates much of the bias encountered with "hard calculated" Scatchard curves. The computer assisted method, in many instances, also results in binding parameters of questionable value. In these cases, the iterative limits on the program must then be adjusted to obtain reasonable results.

In the final analysis, the binding estimate of most use is the maximum binding percentage. This value is accurately measured and involves no interpretation as to its meaning. The maximum binding percentage is actually the product of both the affinity and number of receptors. While the number and affinity of the receptor is intellectually interesting, it is of little value when attempting to interpret binding data; the importance of binding data is not the receptor numbers or affinity but how much hormone will be bound at a given hormone concentration. Additionally, the assumptions used in calculating binding affinities and receptor numbers are generally invalid.

It appears that endocrine research in farm animals still holds much promise. However, the methods presently available for these studies are

inadequate. Therefore, to properly evaluate the hormone status of an animal, the following techniques need to be developed or improved: 1) Estimates of hormone concentrations in plasma need to be integrated over at least a 6 hour period, i.e., continuous sampling techniques. An alternative approach to sampling under physiologic conditions would be the development of very small capillary tubing, which when placed in a vein and attached to a vacuum would continuously sample over an extended period of time. 2) Methods of collecting tissue suitable for hormone sensitivity studies must be developed. These techniques should be applicable to either postmortem tissues or small biopsy tissues. Emphasis should be placed on maintaining intact, viable cells for subsequent assays. 3). Sensitive and specific in vitro measurements of hormone actions at the tissue level must be developed. These should include a physiological event, such as substrate uptake or protein metabolism. 4) Improved methods for assessing hormone binding should be developed. These assays should only be conducted when altered hormone sensitivity is demonstrated. With these techniques, receptor or postreceptor changes could be distinguished. Together these techniques should allow complete description of an animal's hormone status and determine if a hormone is involved in a particular metabolic event (i.e., lipid deposition). In turn, these data should better facilitate attempts to modify growth of farm animals by altering the endocrine system.

APPENDIX

Isolation of bovine mononuclear cells

1. 300 ml blood + 20 ml of isotonic EDTA (4.69 g/100 ml) + 20 ml isotonic MOPS (3.68 g/100 ml).

2. Centrifuge at 1500 G for 15 min in swinging bucket rotor at 4C.

3. Buffy coat is aspirated with pasteur pipet into 20 ml of 25 mM assay buffer:

   25 mM assay buffer (adjusted to pH 7.43 with NaOH)

	g/l
MOPS (25 mM)	5.23
NaCl (105 mM)	6.13
KCl (5 mM)	.37
$MgSO_4$ (1.27 mM)	.15
NaAcetate (15 mM)	1.23
Glucose (6 mM)	1.08
EDTA (1 mM)	.37
Albumin (1 mg/ml)	1.00

4. The buffy coat-buffer suspension is carefully layered over 10 ml of Ficoll-Hypaque gradient with a plastic syringe and a 6"-12 g needle:

   Mononuclear cell isolation gradient (1.075 g/ml, 285 mOsm/l)

Ficoll (21 g + 100 g H_2O)	58 ml
Hypaque-50%	12 ml
Assay buffer (25 mM)	24 ml
H_2O	6 ml

5. Centrifuge gradient + cells for 40 min at 400 g at 18C in a swinging bucket rotor.

6. Mononuclear cells (white layer just above red cells) are carefully removed with a pasteur pipet and diluted with 30 ml of ice cold 25 mM assay buffer.

7. Mononuclear cells are centrifuged 16 min at 5C at 250 G - platelets and broken cells are left in suspension.

8. The supernate is quickly aspirated and cells diluted in fresh ice cold 25 mM assay buffer. Cells were suspended by gentle aspiration and expression with a pasteur pipet.

9. Two additional centrifugations at 250 G for 8 min are carried out at 5C.

10. The cell pellet is resuspended in 5-15 ml of 100 mM assay buffer depending on the cell pellet size:

    100 mM assay buffer composition (pH 7.43); pH adjusted to 7.43 with NaOH

	g/l
MOPS (100 mM)	20.93
NaCl (40 mM)	2.34
KCl (5 mM)	.33
MgSO4 (1.2 mM)	.15
Na Acetate (15 mM)	1.23
Glucose (6 mM)	1.08
EDTA (1 mM)	.37
Albumin (1 mg/ml)	1.00

11. Cells/ml were determined using a hemocytometer.

12. Cells are diluted to approximately 2.5 x 10^7 cells/ml in 100 mM AB.

13. .5 ml of this suspension is added to each tube for binding assays.

Plasma membrane isolation

1. 16 g tissue + 150 ml of ice cold .25 M sucrose. Adipose tissue is homogenized at 37°.

2. Homogenize 90 sec with polytron set at 2 or 3 (minimum speed possible).

3. Homogenate spun 650 G for 10 min at 5°C.

4. Aspirate floating fatty material (when adipose tissue is used, then fatty material must be scraped off).

5. Decant supernate into 50 ml centrifuge tubes.

6. Centrifuge supernate at 10,000 G for 10 min at 5°C.

7. Aspirate fat and decant supernate into clean tubes.

8. Adjust volume to 40 ml with .25 M sucrose.

9. Add 1 ml of NaCl (23.4 g/100 ml) and .5 ml of $MgSO_4$ (0.193 g/100 ml). Final Na and Mg concentrations of .1 M and .2mM respectively.

10. Centrifuge at 37,000 G for 45 min at 5°C.

11. Decant supernates and resuspend pellet in .1 M $NaHCO_3$.

12. Repeat 37,000 G centrifugation 3 times.

13. The final pellet is suspended in 5 mM $NaHCO_3$ and stored at -20C until assayed.

Binding assay procedure for insulin and GH

1. 50 µl - labeled hormone (20-60,000 cpm)
   50 µl - unlabeled hormone
   500 µl - cell or membrane suspension

2. Hormone stock solutions

   Insulin - 1 mg/ml in + .01 N HCl + 7.33 ml AB
   final concentration = 120,000 ng/ml

   GH - .05 mg/.5 ml H_2O + 3.66 ml AB
   final concentration = 120,000 ng/ml

3. Dilutions - Insulin and GH

1st dilution	2nd dilution	ng/ml in assay tube
none	----	10,000
1/9	----	1,000
	1.5/0.5	750
	1.0/1.0	500
	0.5/1.5	250
	----	100
1/9	1.5/0.5	75
	1.0/1.0	50
	0.5/1.5	25
1/9	----	10
	1.5/0.5	7.5
	1.0/1.0	5.0
	0.5/1.5	2.5
1/9	----	1.0
	1.5/0.5	.75
	1.0/1.0	.50
	0.5/1.5	.25
---	----	0

4. After 6 hr at 20°C 3 aliquots of 150 µl each are layered over 200 µl of ice cold AB in microfuge tubes.

5. Centrifuge cells 1 min and membranes 1.5 min in microfuge (Beckman).

6. Separate bound hormone by inverting the microfuge tube and sharply snapping the tube downward with the arm and wrist. The cell pellet will be cleanly separated from the supernate.

7. Inverted tubes can be stored on the bench by using double stick tape until cutting off the tip of the tube containing sedimented membranes.

Representative insulin and GH binding assay

ng/ml hormone	GH bound to Liver (counts/4 min)	Insulin bound to mononucler cells (counts/4 min)
0	1234	8606
.25	1318	7871
.50	1208	7907
.75	1190	8765
1.0	1170	8407
2.5	----	7981
5.0	987	8212
7.5	942	7985
10	896	7477
25	828	7101
50	780	6808
75	729	6814
100	712	5590
250	620	5274
750	610	4656
1000	620	4200
10,000 (NSB)	616	3265
Free	62,000	339,000
cells or protein	170 ng	26 x 10^6/ml

SAS computer program used to analyze Scatchard binding parameters. Variables B/F (BF in program) and Bound (B in program) need to be entered into the program.

Two class model:

```
PROC NLIN METHOD=MARQUARDT BEST=10 CONVERGENCE=.001;  BY AV_NO;
PARMS K1=.05,.1,.3 K2=.001, .0025  R1=.1,.3  R2=5.10;
MODEL BF=.5*((K1*(R1-B)+K2*(R2-B)+SQRT((K1*(R1-B)-K2*(R2-B))**2
++4*K1*K2*R1*R2)));
DER.K1=.5* ((R1-B)+.5* ((K1*(R1-B)-K2*(R2-B))**2
++4*K1*K2*R1*R2)**-.5 *(2* (K1*(R1-B)-K2*(R2-B))*(R1-B) +4*K2*R1*R2));
DER.K2=.5* ((R2-B)+.5* ((K1*(R1-B)-K2*(R2-B))**2
++4*K1*K2*R1*R2)**-.5 *(2* (K1*(R1-B)-K2*(R2-B))*(B-R2) +4*K1*R1*R2));
DER.R1=.5* ( K1   +.5* ((K1*(K1-B)-K2*(R2-B))**2
++4*K1*K2*R1*R2)**-.5 *(2* (K1*(R1-B)-K2*(R2-B))*(K1   ) +4*K1*K2*R2));
DER.R2=.5* ( K2   +.5* ((K1*(R1-B)-K2*(R2-B))**2
++4*K1*K2*R1*R2)**-.5 *(2* (K1*(R1-B)-K2*(R2-B))*(K2   ) +4*K1*K2*R1));
OUTPUT OUT=CORR PREDICTED= BF2;
```

Negative cooperative model:

```
PROC NLIN METHOD=MARQUARDT BEST=10 CONVERGENCE=.001;    BY AV_NO;
PARMS K=.003, .001, .005 R=2, 10, 20 A=.02, .06, .12;
MODEL BF=((K*R)-(K*B))/(1+(((1-A)/A)*B/R));
DER.K=(R-B)/(1+(((1-A)/A)*B/R));
DER.A=((A*R+B-A*B)*(K*R*R-K*B*R)-(A*K*K*R-A*K*R*B)*(R-B))/((A*R+B-A*B)**2);
DER.R=((A*R+B-A*B)*(2*A*K*R-K*A*B)-(K*A*A*R*R)+(K*B*A*A*R))/
((A*R+B-A*B)*(A*R+B-A*B));
OUTPUT OUT=CORR PREDICTED=BFNEG;
```

Miscellaneous calculations

1. Moles of insulin = $.6 \frac{ng}{} \times 10^{13}$

2. Moles of bGH = $2.2 \frac{ng}{} \times 10^{13}$

3. $Kd = \frac{\text{moles hormone (Ro)} \times 1000}{\text{B/F intercept}}$

4. $Ka = \frac{1}{Kd}$

5. $\text{Receptors/cell} = \frac{\text{moles hormone} \times 6.022 \times 10^{23}}{\text{cells/ml}}$